의학석사 학위논문

Characterization of synovial CD16^{+} monocytes affecting inflammatory T-cell response in rheumatoid arthritis.

류마티스에서 염증성 T 림프구에 영향을 미치는 관절활액 CD16^{+} 단핵구의 특성분석.

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윤 보 름

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지도 교수 이 원 우

이 논문을 의학석사 학위논문으로 제출함

2012년 10월

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의학과 미생물학 전공

윤 보 름

윤보름의 의학석사 학위논문을 인준함

2012년 12월

위 원 장 박 정 규 (인)

부위원장 예 상 규 (인)

위 원 이 원 우 (인)

Abstract

Monocytes play critical roles in the pathogenesis of chronic inflammatory diseases, including autoimmunity as well as in the innate response to infections. Human monocytes are heterogeneous and generally classified into three subsets based on CD14 and CD16 expression. Although increasing evidences suggest that three monocyte subsets have distinct functions in inflammatory conditions, little is known about their roles in pathogenesis of autoimmune diseases. To address this issue, I investigated the phenotypic and functional characteristics of monocytes in the synovial fluid (SF) and peripheral blood (PB) from patients with rheumatoid arthritis (RA). CD16 expression on $CD14^{+}$ monocytes in the SF was significantly increased compared with the PB of RA patients and healthy controls, whereas $CD14^{dim}CD16^{+}$ monocytes were rarely observed in the SF. The majority of synovial monocytes constitutively expressed CD80, while peripheral monocytes did not express CD80 without stimulation. In addition, the expression of membrane-bound IL-15 was markedly elevated in the RA patients. The TLR expression between peripheral monocyte and synovial monocytes were distinct, monocytes from synovial fluid had increased TLR levels generally. To explore how synovial monocytes gain unique properties, PB monocytes were stimulated with various cytokines and TLR ligands. Interestingly, TGF-β is a potent inducer of CD16 expression on $CD14^{+}$ monocytes, whereas expressions of CD80 and membrane-bound IL-15 were significantly elevated by IFN-γ exerting a synergistic effect with IL-15. The synovial monocytes were found to significantly promote

Th17 and Th1 responses *in vitro*, compared with PB monocytes from RA patients. The findings in this study suggest the possible role for cytokine milieu of the SF in giving unique features to synovial monocytes and their important roles in affecting inflammatory T-cell response in RA.

Key words ; CD16 monocytes, Rheumatoid arthritis, Th17, TGF-β

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Introduction

Monocytes are circulating mononuclear phagocytes that have been generally considered as systemic precursors for tissue macrophages and inflammatory dendritic cells (DCs). Blood monocytes play critical roles in host defense against infection of microbes, fungi, virus and parasites by phagocytosis and production of reactive oxygen species (ROS) and secrete proinflammatory cytokines such as, TNF-α, IL-6, and IL-1β (1). More recently, monocytes are also implicated in various inflammatory diseases, including atherosclerosis (2).

In mice and humans, monocytes are classified into two major populations on the basis of their phenotypes. Traditionally, human monocytes have been defined as cells that express CD14 which is co-receptor of lipopolysaccharide (LPS). However Ziegler-Heitbrock and colleagues identified a minor population of human monocytes which express CD16 (FcγRIII) and demonstrated differential functionality between two $CD14^{+}$ and $CD16^{+}$ monocytes.(3) On the basis of *in vivo* mouse studies, murine monocytes are divided into two subsets; $LY6C^{high}$ ($Gr1^{+}$) and $LY6C^{low}$ ($Gr1^{-}$) monocytes. The former population specializes in proinflammatory and antimicrobial roles, whereas the latter population involve in patrolling the blood vessel lumen by associating with the vascular endothelium and in promoting wound healing (4-6). Although two monocyte populations in human and mice are not precisely overlapping, their contribution to immune responses seems to be similar (7). Human $CD14^{+}$ monocytes, which are called “classical monocytes”, represent 80-90% of blood monocytes and express high level of

chemokine receptor CCR2 and low level of CX3CR1. Their phenotype and function resembles those of the murine LY6C^{high} (Gr1^{+}) monocytes. In contrast to CD14^{+} monocytes, CD16^{+} monocytes are characterized by CX3CR1^{high}CCR2^{low} and are similar to the murine LY6C^{low} (Gr1^{-}) monocytes which are called "patrolling monocytes" (8, 9).

More recently, human monocytes expressing CD16 are suggested to be subdivided further into two subsets, CD14^{+}CD16^{+} and CD14^{dim}CD16^{+} monocytes, on the basis of level of surface CD14 expression, their gene expression profiles, and responses against external stimulus (1). The CD14^{+}CD16^{+} monocytes share many features of "classical" CD14^{+}CD16^{-} monocytes having high capacity of phagocytosis and producing pro-inflammatory cytokines in response to ligand of TLR2 and TLR4. Meanwhile, the CD14^{dim}CD16^{+} monocytes rarely show capacity of phagocytosis but selectively produce TNF-α and IL-1β in response to virus or immune complexes containing nucleic acid stimuli, through TLR7 and TLR8 mediated signaling pathway (1). Of importance, CD16^{+} monocytes including CD14^{+}CD16^{+} and CD14^{dim}CD16^{+} monocyte populations expand in peripheral blood of patients with acute and chronic inflammation (10-12) but the actual function of the CD16^{+} monocytes still remains elusive.

There is accumulating evidence that inflammatory monocytes could play important roles in induction and shaping of T-cell responses (6, 10). Indeed, several studies have recently demonstrated that TLR-stimulated monocytes are required for optimal induction of IL-17-producing CD4 T-cells (Th17 cells) which have critical

roles of pathogenesis of autoimmune diseases. These finding indicate that the monocytes are closely linked to regulation of the adaptive immune responses (13, 14). It has been well documented that CD4 helper T cells (hereafter Th17 cells) selectively producing IL-17 are linked to many chronic inflammation and autoimmune diseases, including multiple sclerosis (MS), psoriasis, inflammatory bowel diseases (IBD), and rheumatoid arthritis (RA) (15). In humans, *in vitro* Th17 differentiation requires stimulation via CD3 and CD28 in the presence of IL-1β, IL-6, and IL-23, whereas murine Th17 cells can be induced by CD3/CD28 stimulation with TGF-β and IL-6 alone. Human monocytes stimulated through certain TLRs produce high levels of IL-1β, IL-6, and IL-23 and the environment milieu by these cytokines can have important effects on the polarization and expansion of T cells as antigen-presenting cells (APCs) do (13, 16).

Rheumatoid Arthritis (RA) is representative systemic autoimmune disease and chronic inflammatory disease that targets synovial membrane (14). There have been many studies about pathogenesis of RA, several candidates are shown up like genetic factors, infection and abnormal secretion of hormones, but the exact immune mechanism still remains unclear. Since the frequency of $CD16^+$ monocytes in RA patients is significantly elevated and they produce enormous amounts of TNF-α in response to TLR2 stimulation (9, 17), $CD16^+$ monocytes are implicated in pathophysiology of RA but their pathogenic roles need to be clear, especially their modulatory roles for T-cell responses. Of important, the $CD16^+$ monocytes expressing high level of surface CX3CR1 are seemed to migrate to synovium by

CX3CR1-CX3CL1 interaction (18, 19).

Although increasing evidences suggest that inflammatory $CD16^+$ monocytes have distinct functions in inflammatory conditions, little is known about their roles in pathogenesis of autoimmune disorders including RA. To address this issue, I investigated the phenotypic and functional characteristics of monocytes in the synovial fluid (SF) and peripheral blood (PB) from patients with RA and healthy controls. In present studies, my findings suggest the possible role for cytokine milieu of the SF in giving unique features to synovial monocytes and their important roles in affecting inflammatory T-cell response in RA.

Material and Methods

Cell isolation from healthy controls (HC) and RA patients

Peripheral blood and synovial fluid of RA patients were obtained from the Department of Internal Medicine, Chungnam National University Hospital. Peripheral blood of healthy volunteers was drawn after obtaining informed consent at Seoul National University College of Medicine (IRB No.1109-055-378). Mononuclear cells were isolated from peripheral blood or synovial fluid by density gradient centrifugation (Bicoll separating solution; BIOCHROM Inc, Cambridge, UK). Monocytes were positively separated with CD14 microbeads (Miltenyi Biotec Inc, Auburn, CA) from peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC). CD4 memory T cells were negatively purified with human memory CD4+ T cell enrichment kit (EasySep negative selection; STEMCELL technologies Inc, Vancouver, Canada).

Flow cytometric analysis

PBMC and SFMC were stained for 30min at 4 degree with anti-CD3-allophycocyanin (APC), anti-CD14-APC, anti-CD19-APC, anti-CD56-APC, anti-CD3-APC-cyanin 7 (Cy7), anti-CD14-APC-Cy7, anti-HLA-DR-fluorescein isothiocyanate (FITC), anti-CD68-phycoerythrin (PE), anti-CD80-PE, anti-CD86-PE, anti-CD16-PE- cyanin 5 (Cy5), anti-CD4-PE-Cy5, (all from BD Bioscience, San Jose, CA), anti-CX3CR1-PE, anti-TLR4-PE (all from eBioscience Inc, San Diego, CA), anti-CCR2-PE, anti-IL-15-PE (all from R&D systems, Minneapolis,

MN), PE-TLR7 (IMGENEX corp, San Diego, CA), and PE-TLR8 (Abcam plc, Cambridge, MA).Stained cells were acquired by a BD LSRII (BD bioscience) and analyzed by using Flowjo software (ver. 9.0; Tree star, OR).

Cell culture

Purified monocytes and CD4 memory T cells from HC and RA patients were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine. Purified monocytes were seeded at 5×10^3 into U-bottomed 96-well plate in the presence of soluble anti-CD3 antibody (1μg/ml; BD bioscience), anti-CD28 antibody (1μg/ml; BD bioscience), and LPS (100ng/ml; Sigma-Aldrich Inc, St. Louis, MO). After 1hr incubation, 2.5 X 10^4 CD4 memory T cells were added into each well, and co-cultured with LPS-activated monocytes for 7 days. In some experiments, purified monocytes were treated with TGF-β (10ng/ml; R&D systems) in the presence or absence of recombinant human IFN-γ (25ng/ml; ebioscience) and/or recombinant human IL-15 (25ng/ml; R&D systems) in 5ml polypropylene tube (BD falcon) for 18hrs, followed by FACS staining with antibodies to CD14, CD16, and HLA-DR. For monocytes treated with various cytokines, RPMI 1640 medium supplemented with 10% human AB serum was used to avoid any stimulation of monocytes with FBS.

Intracellular cytokine staining (ICS) and enzyme-linked immunosorbent assay (ELISA)

For intracellular cytokine staining, CD4 T cells co-cultured with monocytes for 7

days were re-stimulated 6 hours with PMA (50ng/ml; Sigma-Aldrich) and ionomycin (1ug/ml; Sigma-Aldrich) in the presence with Golgiplug (BD bioscience) for last 4 hours. Cells were fixed and permeablized with BD Cytofix/Cytoperm™ kit. Cells were stained with antibodies to anti-IL-17A-PE, anti-IFN-γ-PE-Cy7, anti-IL-6-PE (all from eBioscience), anti-TNF-α-V450 (BD bioscience), and anti-IL-1β-Alexa 647 (Biolegend Inc, San Diego, CA) followed by analysis on a BD LSRII. In some experiments, intracellular cytokine staining was performed with freshly isolated PBMC and SFMC from RA patients. Various cytokines (IL-6, IL-1β, IL-17A, IFN-γ, and TNF-α) in co-culture supernatant were quantified by commercial ELISA kits (eBioscience for IL-6, IL-1β, IL-17A, and TNF-α and Biolegend for IFN-γ). The measurement of OD (Optical density value) was performed using DTX 880/multimode detector (Beckman coulter).

Quantitative RT-PCR

Total RNA was extracted from freshly isolated or co-cultured cells using TRIzol reagents (Invitogen by life technologies corp, Grand Island, NY) and cDNA was synthesized by GoScript reverse transcription system (Promega corp, Madison, WI). Primers were designed using the OligoPerfect Designer (Invitrogen; sequences in Table 1) or adopted from previously described primer sequences [Ref]. Real-time quantitative reverse-transcription-polymerase chain reaction (RT-PCR) was performed in triplicate on a 7500 PCR system (Applied Biosystems) using the iQ™SYBR® Green supermix (Bio-Rad Laboratories Inc, Hercules, CA). The levels of gene expression were normalized to the expression of *ACTINB*. The

comparative C_T method ($\Delta\Delta C_T$) was used for the quantification of gene expression.

Table 1. Gene-specific primers used in the real-time RT-PCR reactions :

Gene	*Product size*	*Primer sequence*
CD16A	210bp	Forward 5'- GCT CCG GAT ATC TTT GGT GA-3'
		Reverse 5'-CTC CCT GGC ACT TCA GAG TC-3'
CD68	152bp	Forward 5'-ACT GAA CCC CAA CAA AAC CA-3'
		Reverse 5'-TTG TAC TCC ACC GCC ATG TA3'
CD80	215bp	Forward 5'-GGG AAA GTG TAC GCC CTG TA-3'
		Reverse 5'-GCT ACT TCT GTG CCC ACC AT-3'
CD86	211bp	Forward 5'-TGG AAC CAA CAC AAT GGA GA-3'
		Reverse 5'-GGT TGC CCA GGA ACT TAC AA-3'
CX3CR1	198bp	Forward 5'-GCA AGA AGC CCA AGA GTG TC-3'
		Reverse 5'-ATG CTG ATG ACG GTG ATG AA-3'
IL-15Rα	212bp	Forward 5'-ACC TTC CAC AGG AAC CAC AG-3'
		Reverse 5'-AGG TAG CAT GCC AGG AGA GA-3'
TLR2	176bp	Forward 5'-ATT GTG CCC ATT GCT CTT TC-3'
		Reverse 5'-CTG CCC TTG CAG ATA CCA TT-3'
TLR4	198bp	Forward 5'-TC TTC AAC CAG ACC TCT ACA TTC CA-3'
		Reverse 5'- GGA ACA TCC AGA GTG ACA TCA CAG-3'
TLR7	207bp	Forward 5'-TCA CTC CAT GCC ATC AAG AA-3'
		Reverse 5'-ACC ATC TAG CCC CAA GGA GT-3'
TLR8	111bp	Forward 5'-TTT TCT TCA TTG GGC CAA AC-3'
		Reverse 5'-GAA TGG CTG AAA ATT CAG TTC C-3'

Statistical analysis

A paired *t*-test was done to analyze data using Graph pad Prism 5 (GraphPad Software Inc, La Jolla, CA) and Microsoft Excel 2010. *P* values of less than .05 were considered statistically significant.

Results

Increased expression of CD16 on monocytes of RA patients

To investigate whether the frequency of $CD16^+$ monocyte is changed in RA patients enrolled in this study, I first analyzed mononuclear cells isolated from peripheral blood (PB) and synovial fluid (SF) of RA patients or healthy controls by multicolor flow cytometry. Since majority of human NK cells and B cells also express CD16 and HLA-DR on their surface, respectively, flow cytometric analysis was carefully performed by following gating strategy (Fig. 1). Monocytes were identified on the basis of their characteristic forward- and side-scatter profile. Within this gate, monocytes were defined as HLA-DR^+ cells which do not express B cell (CD19), T cell (CD3), or NK cell (CD56) lineage markers. Among the HLA-DR^+ cells, three human monocyte populations were well separated by CD14 and CD16 expression (Fig. 1A). In healthy controls, approximately 85% of PB monocytes represented "classical" $CD14^+CD16^-$ monocyte population, whereas the frequency of $CD16^+$ monocytes was under 10% of total PB monocytes. Consistent with previous reports (1, 3, 11), the $CD16^+$ monocytes in healthy controls could be further subdivided into "inflammatory" or "intermediate" $CD14^+CD16^+$ (62.80 % of $CD16^+$ monocytes) and "nonclassical" or "patrolling" $CD14^{dim}CD16^+$ (37.20 % of $CD16^+$ monocytes) (Fig. 1A and 2A). The frequency of $CD16^+$ monocytes (15.52 ± 10.79 %) was significantly increased in peripheral blood of RA patients compared to healthy controls (8.60 ± 5.34 %). More importantly, this change was due to the expansion of $CD14^+CD16^+$ population (12.77 ± 10.27 % versus 5.29 ±

3.75%) but not $CD14^{dim}CD16^{+}$ population (2.75 ± 2.50 % versus 3.31 ± 3.0 %). The frequency of PB $CD14^{dim}CD16^{+}$ monocytes in RA patient was rather slightly decreased compared to healthy controls thought it was not statistically significant (Fig 1B and 2A).

In synovial fluid, $CD14^{+}CD16^{-}$ monocytes were significantly reduced when compared to peripheral blood of same patients (59.90 ± 22.57 % versus 82.06 ± 11.28 %; $p<0.004$). This reduction seems to be associated with increase of $CD14^{-}CD16^{-}$HLA-DR^{+} cells as well as $CD14^{+}CD16^{+}$ monocytes. Increase of $CD16^{+}$ monocytes (23.15 ± 15.22 %) were more markedly observed in synovial fluid of RA patients. There was a trend towards higher frequency of $CD16^{+}$ monocyte in synovial fluid than that in peripheral blood of same patient ($p=0.11$; 12 of 15 patients) (Fig 1B and 2A). Among SF $CD16^{+}$ monocytes, the proportion of $CD14^{+}CD16^{+}$ monocytes (88.73 ± 9.41 %) was substantially higher than that in peripheral blood of RA patients (75.62 ± 22.70 %) or healthy controls (63.80 ± 25.18 %), suggesting that increase of SF $CD16^{+}$ monocytes mainly results from expansion of $CD14^{+}CD16^{+}$ monocytes. Increased expression of CD16 on SF monocytes was confirmed at gene level by real time RT-PCR. The mRNA level of the CD16 gene were higher in purified $CD14^{+}$ monocytes from synovial fluids than those from peripheral blood of same RA patient (Fig. 2A).

Since several studies revealed that $CD16^{+}$ monocytes expanded in inflammatory conditions, I next asked whether the frequency of $CD14^{+}CD16^{+}$ monocytes correlated with known RA disease parameters such as DAS28, ESR, and CRP. No

association with the parameters was observed in present study so far.

Phenotypic characteristics of PB and SF monocytes from RA patients

As mentioned previously, different monocyte subsets have characteristic chemokine receptor and regulatory molecule expression profiles, indicating their differential migratory capability and functionality (8, 9). To investigate expression profiles of the surface molecules, including chemokine receptors, co-stimulatory molecules, and etc, on monocytes of RA patients, the expression profiles were compared among PB monocytes from healthy or RA patients and SF monocytes from RA patients using multicolor flow cytometric analysis.

Consistent with previous reports (4, 7), $CD14^{+}$ monocytes in healthy control express higher CCR2 and lower but still considerable level of CX3CR1. In the case of $CD14^{dim}CD16^{+}$ monocytes, $CX3CR1^{high}CCR2^{-}$ phenotype was observed. Co-stimulatory molecule CD86 was constitutively expressed at high level but CD80 was barely expressed on PB monocytes of healthy controls (Fig 3A). When compared to monocytes of healthy controls, CX3CR1 expression in RA patients was significantly decreased in PB $CD16^{+}$ monocytes and even more decreased in SF $CD16^{+}$ monocytes. Especially, CX3CR1 expression levels of SF monocytes in RA patients were very similar among three monocyte populations. CCR2 and co-CD86 expression levels on monocytes did not significantly differ between healthy controls and RA patients. Consistent with FACS analysis, the gene expression of CX3CR1 on RA SF monocytes was reduced at 50% (Fig 3B-D).

The most outstanding difference was the expression of co-stimulatory molecules CD80 on SF monocytes but not on PB monocytes of RA patients. As abovementioned, PB monocytes did not express CD80 in healthy control and even in RA patients (Fig. 4A). Elevated CD80 expression was a unique feature of SF monocyte of RA and this induction was confirmed at gene expression level by real-time RT-PCR (Fig. 4B). CD80 gene expression of SF monocyte was around 40 folds higher than that of PB monocytes in same RA patients.

HLA-DR was a MHC class II molecule that gives 'first signal' to T cell receptor (TCR) on CD4 T cells. Antigen presenting cells (macrophage, B cells, and dendritic cells) typically express HLA-DR. Upon stimulation, increased level of HLA-DR expression was observed in antigen presenting cells as well as various cells including T cells. Of importance, enhanced HLA-DR expression was seen on SF monocytes (Fig. 4C) and this increase was even more marked in $CD14^{+}CD16^{+}$ than $CD14^{+}CD16^{-}$ monocytes (Fig. 4D).

Interleukin-15 (IL-15) is one of the common γ chain cytokines and exists in membrane-bound form usually bound with IL-15 receptor alpha (IL-15Rα) on the surface of monocytes or dendritic cells. The membrane-bound IL-15 (mIL-15) regulates activation and proliferation of T cells and NK cells, and up-regulation of mIL-15 has a central role in the several autoimmune and chronic inflammatory diseases (20). Thus I measured mIL-15 expression on PB monocytes from healthy controls and RA patients and on SF monocytes from RA patients (Fig. 5A, representative data). PB monocytes in RA patients showed a trend toward higher

mIL-15 expression level compared to healthy controls. Intriguingly, mIL-15 expression on monocytes was rather reduced on SF monocytes in RA patients. The level of mIL-15 expression on SF monocytes was comparable to that on PB monocytes of healthy controls (Fig. 5B).

Distinct TLR expression profiles of PB and SF monocytes from RA patients

Toll like receptors (TLRs) are a family of proteins that play critical roles in innate immune system including monocytes. Several studies have recently demonstrated that TLR-stimulated monocytes are required for optimal induction of Th17 cells which are involved in pathogenesis of various autoimmune diseases. Thus I analyzed various TLR expressions on monocytes from blood and synovial fluid of RA patients or healthy controls. Among TLRs tested in this study, TLR8 gene was markedly elevated in PB monocytes in RA patients when compared to healthy controls (Fig. 6A). When compared between PB and SF monocytes in RA patients, the levels of TLR4 gene expression were enhanced by approximately 10 times in monocytes from synovial fluid (Fig. 6B).

Effect of various cytokines which can induce distinct phenotype of monocytes in RA patients

$CD16^+$ monocytes expand in various acute and chronic inflammatory diseases. Certain cytokine milieus have been suggested to induce CD16 on monocytes in inflammatory conditions (21-23). To explore possible mechanism underlying CD16 induction on monocytes, I treated various classes of cytokines and ligands for TLR

on $CD14^+$ monocytes purified from healthy control and analyzed the change of CD16 level. TLR activation with LPS (ligand for TLR4) and R848 (ligand for TLR7 and TLR8) rather down-regulated CD16 expression on monocytes and proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α cytokines, did not change the CD16 expression. Among the cytokines tested, TGF-β treatment for 18 hours clearly elevated CD16 expression on PB $CD14^+$ monocytes of healthy controls (Fig. 7A). Approximately 20% of monocytes induced CD16 molecules on their cell surface compared to no treated monocytes (Fig. 7B). Next I explored cytokines which are able to induce CD80 and mIL-15 on monocytes. Since TGF-β was the key inducer of CD16 on monocytes, other cytokines were treated on monocytes in the presence of TGF-β. The expression of CD80 was significantly increased in purified $CD14^+$ monocytes which were treated with IFN-γ in the presence of TGF-β (Fig. 8). The treatments of TGF-β alone had no effects on expression level of CD80. The expression of mIL-15 was enhanced by treatment with IFN-γ or IL-15 regardless of TGF-β treatment. Of interest, treatment of IL-15 had a synergetic effect on expression of mIL-15 when treated with IFN-γ (Fig. 9A). Monocytes treated with IFN-γ caused not just only increase of mIL-15 expression but also induction of IL-15Rα (Fig. 9B) which can do trans-presentation of mIL-15 to effector cells. These findings suggest that the distinct phenotype of monocytes from RA patients may be induced under certain cytokine milieu.

The SF monocytes significantly promoted Th17 and Th1 responses *in vitro*, compared with PB monocytes from RA patients.

The findings so far clearly showed that SF monocytes in RA patients have distinct phenotype. Next I investigated whether these SF monocytes in RA patient have unique capacity to modulate and shape T-cell responses. To answer this question, $CD14^+$ monocytes (including $CD14^+CD16^-$ and $CD14^+CD16^+$ monocytes) and CD4 memory T cells were highly purified from peripheral blood and synovial fluids in the same patients. Purified PB and SF CD4 memory T cells were criss-cross co-cultured for 7 days with PB or SF $CD14^+$ monocytes in the presence of soluble anti-CD3, anti-CD28 antibodies and LPS (Fig. 10A). Intracellular cytokine staining (ICS) on day 7 showed the frequencies of IL-17A and/or IFN-γ producing CD4 T cells purified from peripheral blood were significantly higher in the culture conditions with SF monocytes than PB monocytes in RA patients (Fig. 10B). Consistent with ICS data, the amount of IL-17A in the culture supernatant was significantly increased when PB CD4 memory T cells were co-cultured with SF monocytes (Fig. 11). In this culture condition, higher amount of IFN-γ was also seen but did not reached to statistical significance. Taken together, monocytes derived from synovial fluid have higher capacity to induce production of cytokines from CD4 memory T cell than derived from peripheral blood.

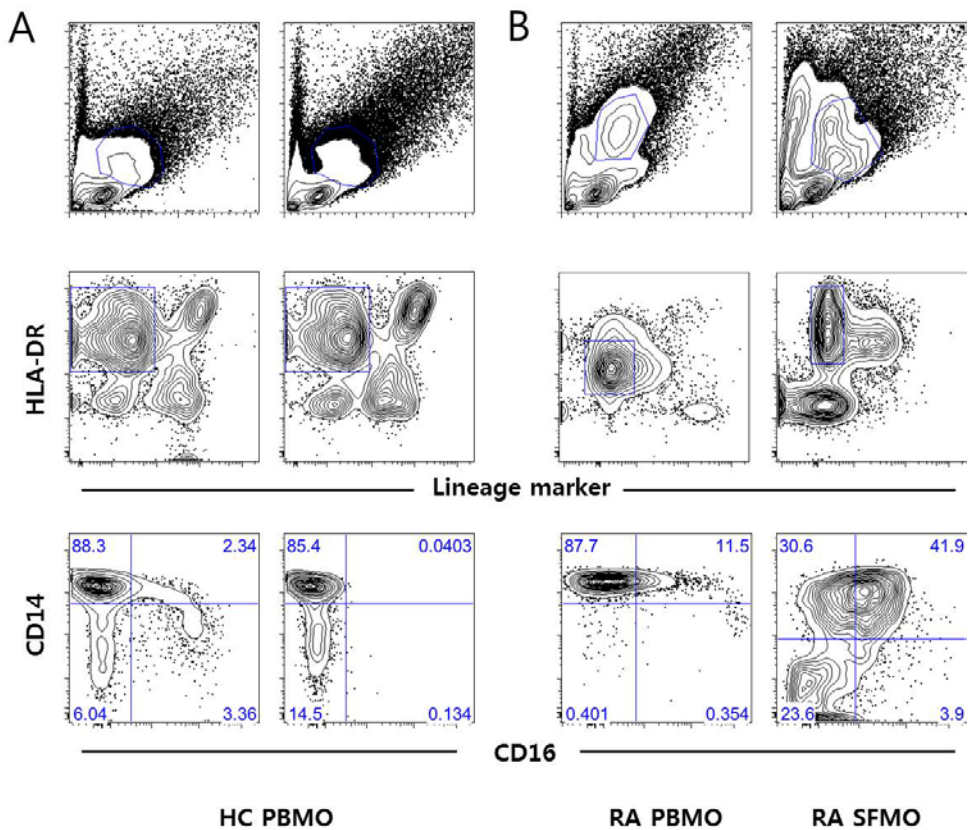


Figure 1. The strategy of gating purified monocytes using flow cytometry.

Flow cytometric analysis of (A) peripheral blood monocytes (PBMO) from healthy controls and (B) monocytes from peripheral blood (left) or synovial fluid (SFMO, right) in RA patients.

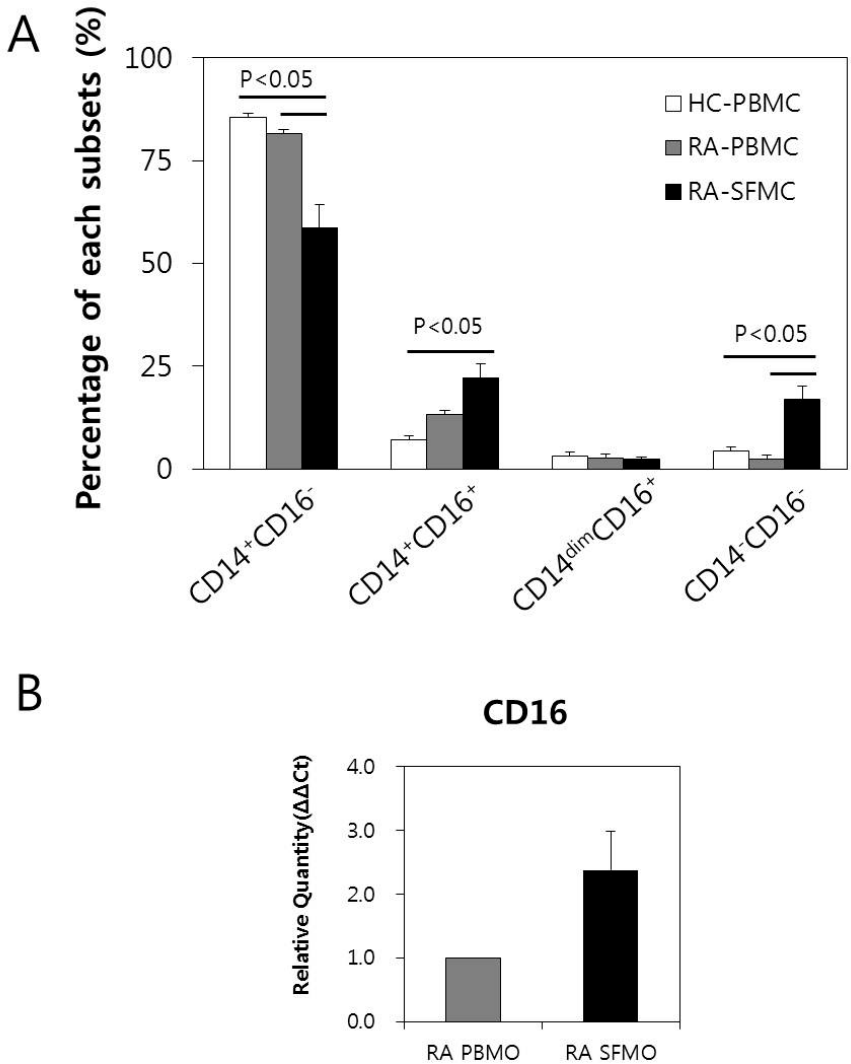
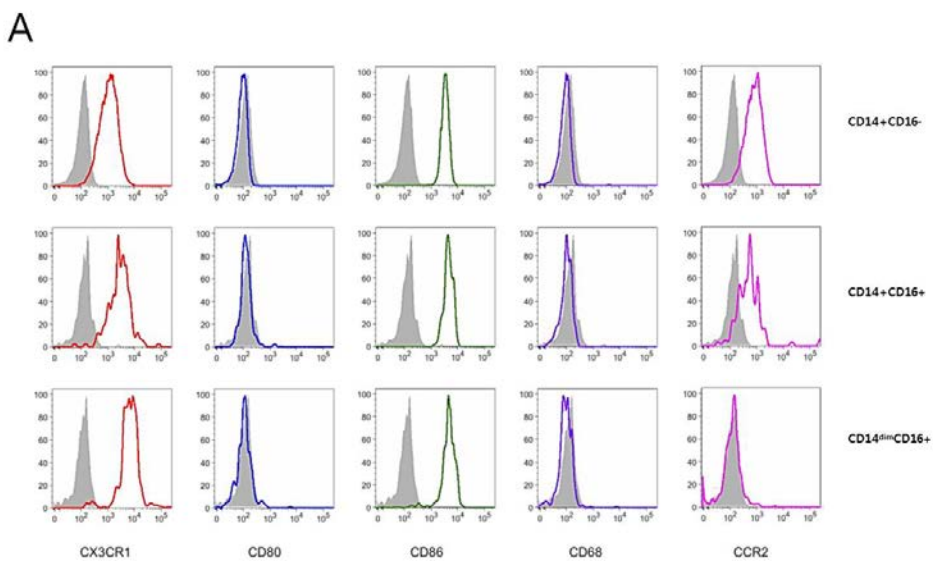


Figure 2. Monocytes derived from synovial fluid of RA patients had increased expression of CD16.

(A) The frequency (%) of each subsets which were subdivided by differential expressions of CD14 and CD16 on monocytes. (B) Quantitative PCR analysis of CD16 expression between PBMO and SFMO in RA patients (n=5).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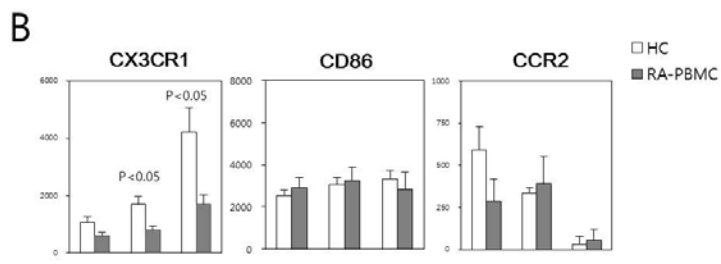
CD14+CD16-
CD14+CD16+
CD14dimCD16+
CX3CR1
CD80
CD86
CD68
CCR2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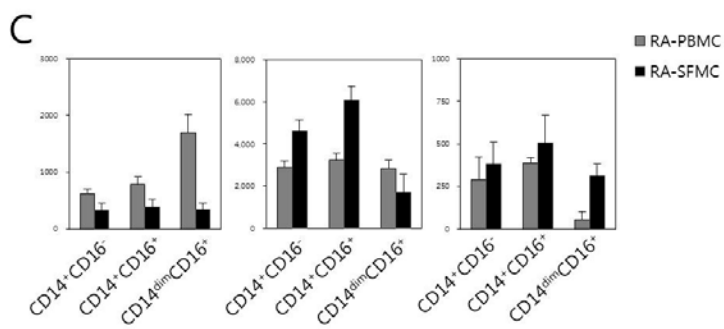
CD86
CCR2
HC
RA-PBMC
P<0.05
P<0.05


C
RA-PBMC
RA-SFMC
CD14+CD16-
CD14+CD16+
CD14dimCD16+

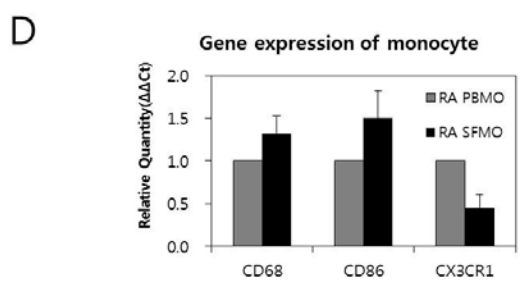

D
Gene expression of monocyte
Relative Quantity(ΔΔCt)
RA PBMO
RA SFMO
CD68
CD86
CX3CR1

Figure 3. The expressions of surface molecules on monocytes derived from RA patients and healthy controls.

(A) Representative flow cytometric analysis of CX_3CR1, CD80, CD86, CD68 and CCR2 expression on monocytes from peripheral blood of healthy control. (B) CX_3CR1, CD86 and CCR2 expression on monocytes from peripheral blood of RA patients and healthy controls and (C) synovial fluid of RA patients. (D) Relative gene expressions of CD68, CD86, and CX_3CR1 in isolated monocytes from peripheral blood and synovial fluid of RA patients by quantitative PCR analysis (n=5).

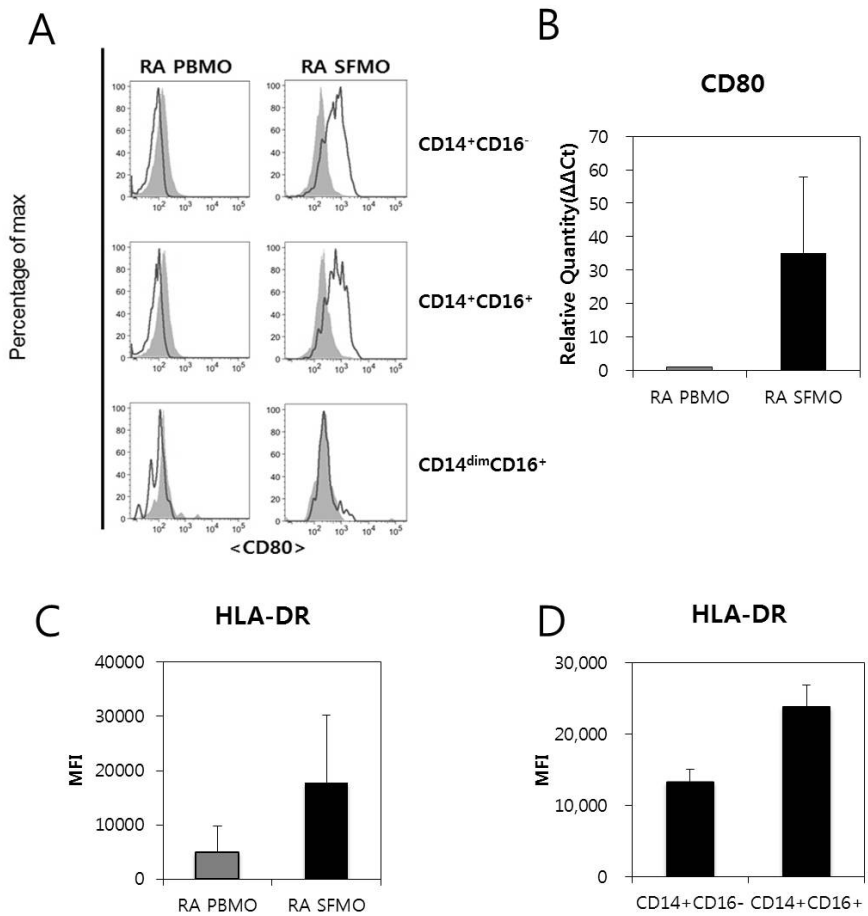


Figure 4. The levels of CD80 and HLA-DR expression were markedly elevated in monocyte derived from synovial fluid of RA patients.

(A) Flow cytometric analysis of CD80 expression among PBMO in healthy controls, PBMO and SFMO in RA patients (Gray shade: isotype control). (B) Relative gene expression of CD80 in isolated monocytes from peripheral blood and synovial fluid of RA patients (n=5). (C) Comparison of MFIs (mean fluorescent intensity) of HLA-DR between PBMO and SFMO in RA patients (n=19), (D) between $CD14^{+}CD16^{-}$ and $CD14^{+}CD16^{+}$ monocytes subsets in SFMO.

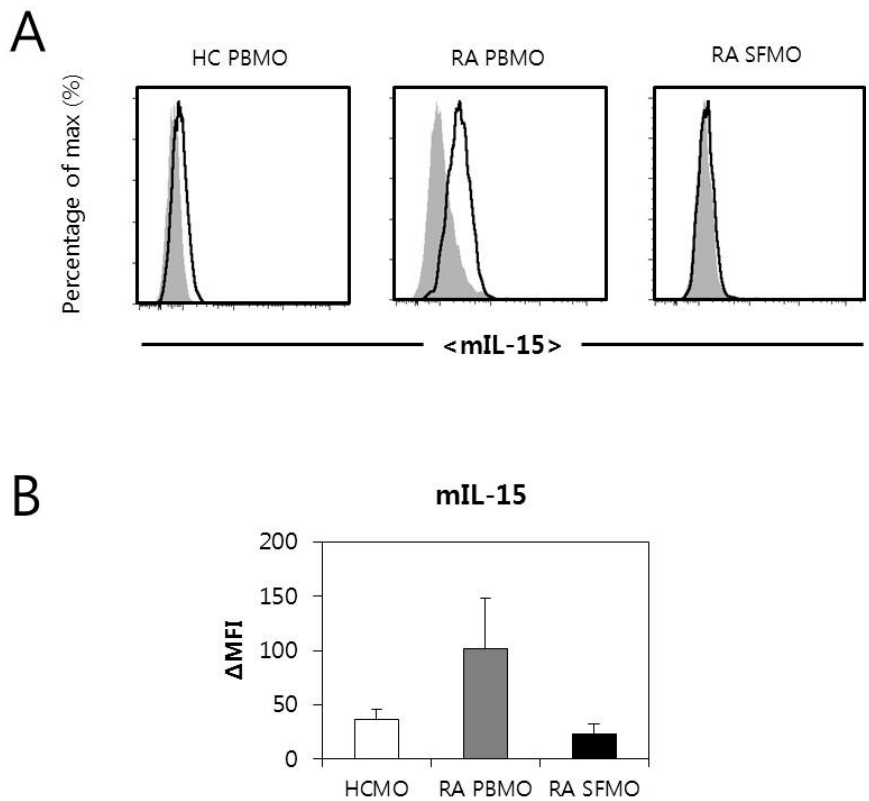


Figure 5. Membrane-bound IL-15 expression on surface of monocytes was elevated in peripheral blood of RA patients.

(A) Membrane-bound IL-15 (mIL-15) expression on monocytes from peripheral blood of healthy controls, peripheral blood and synovial fluid of RA patients. (B) ΔMFI of mIL-15 on monocytes from healthy controls and RA patients.

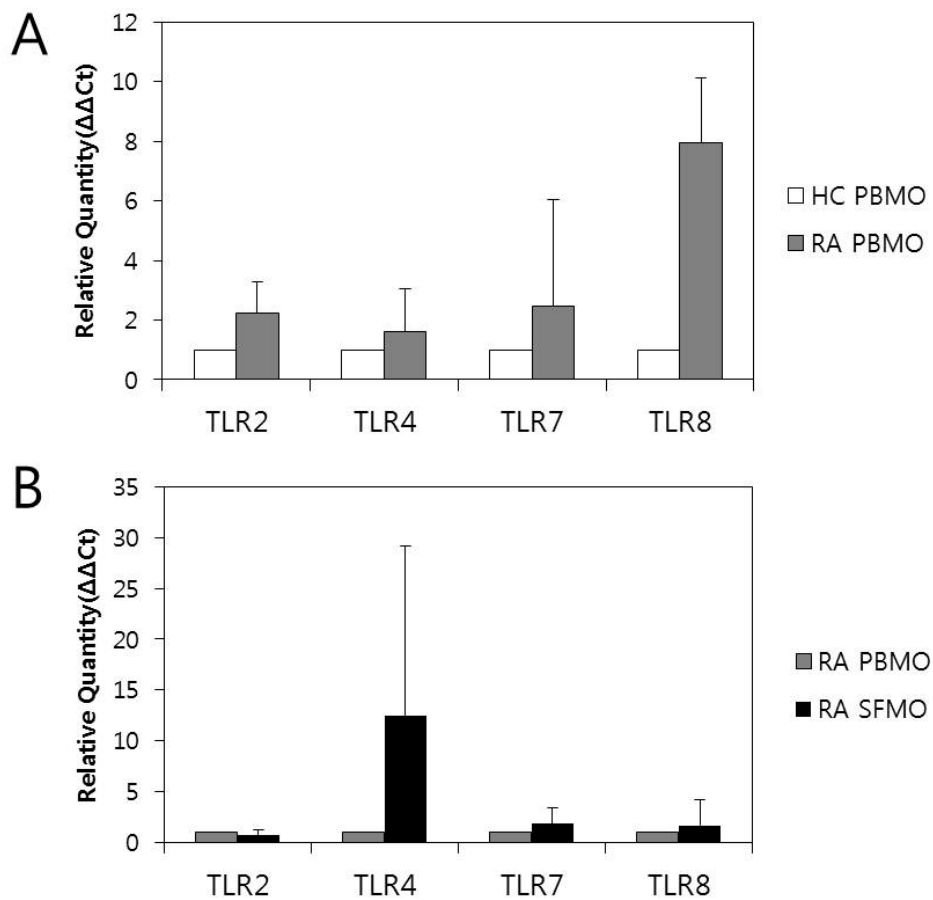


Figure 6. The differential expressions of TLRs in monocytes derived from healthy controls' and RA patients' peripheral blood or synovial fluid.

(A) Relative gene expressions of TLRs in isolated monocytes from peripheral blood of healthy controls' and RA patients', and (B) compared with monocytes from synovial fluid of RA patients'.

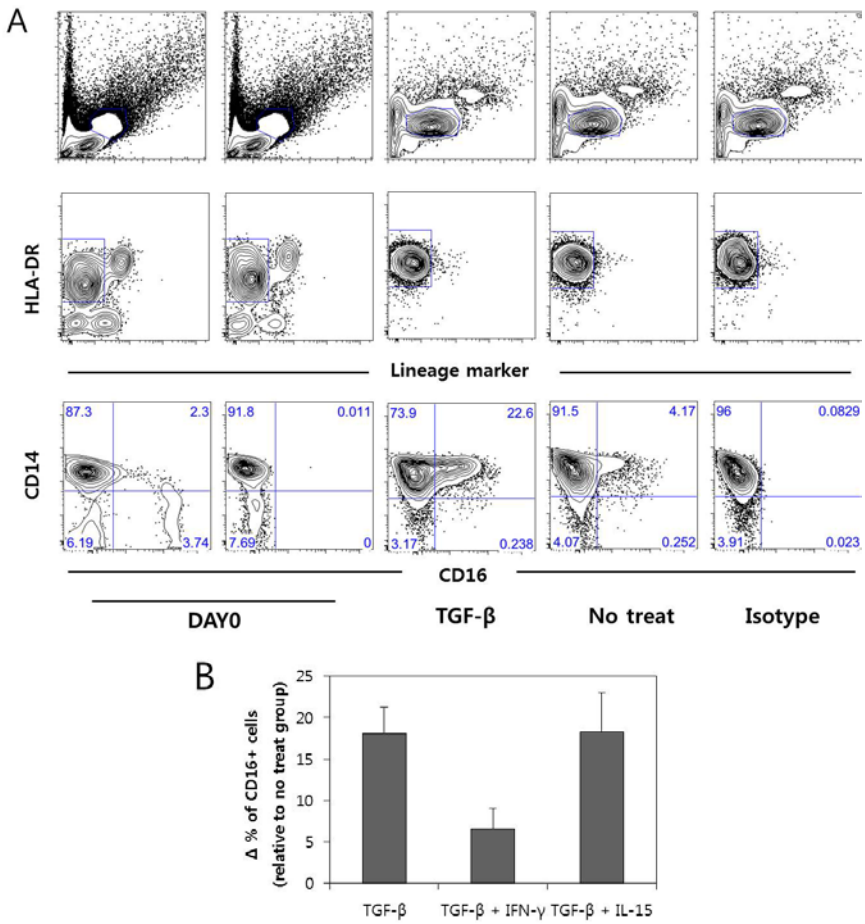


Figure 7. TGF-β treatment induced CD16 expression of monocytes in healthy controls.

Isolated monocytes were treated with TGF-β (10ng/ml) in the absence or presence of other cytokines (IFN-γ or IL-15) for 18 hours in RPMI-1640 medium supplemented with 10% human AB serum. (A) Increased CD16 expression on monocyte after 18 hours-cytokine treatment (DAY0 indicates phenotype of monocyte before cytokine treatment). (B) Change on percentage of $CD16^{+}$ cells in cytokine-treated monocytes.

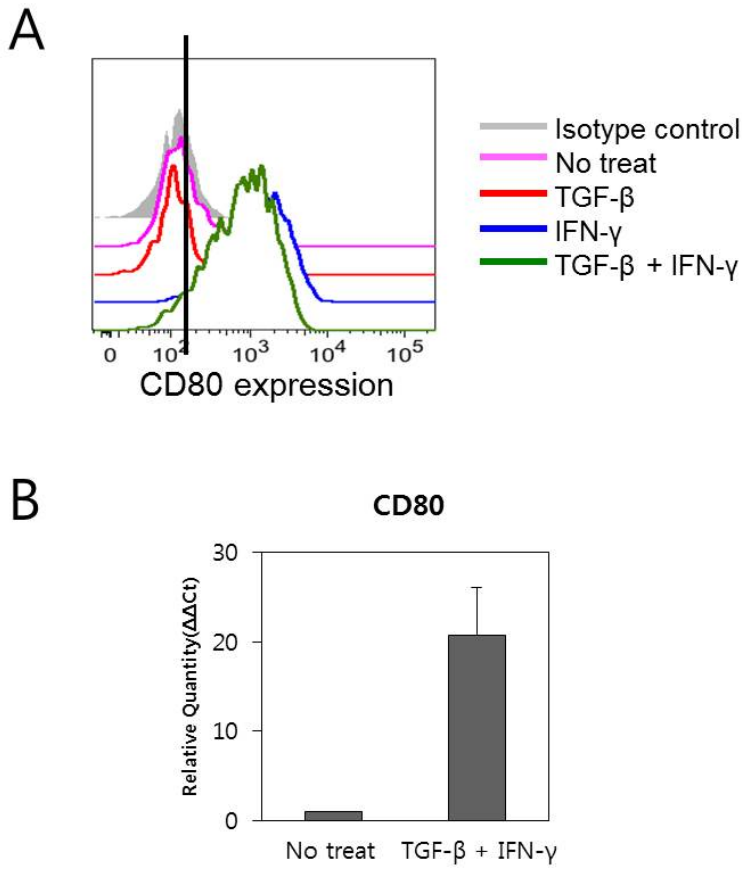


Figure 8. CD80 expression was increased by IFN-γ treatment.

(A) Flow cytometric analysis of CD80 expression on cytokine-treated monocytes

(B) Relative gene expression of CD80 in cytokine-treated monocytes (n=3).

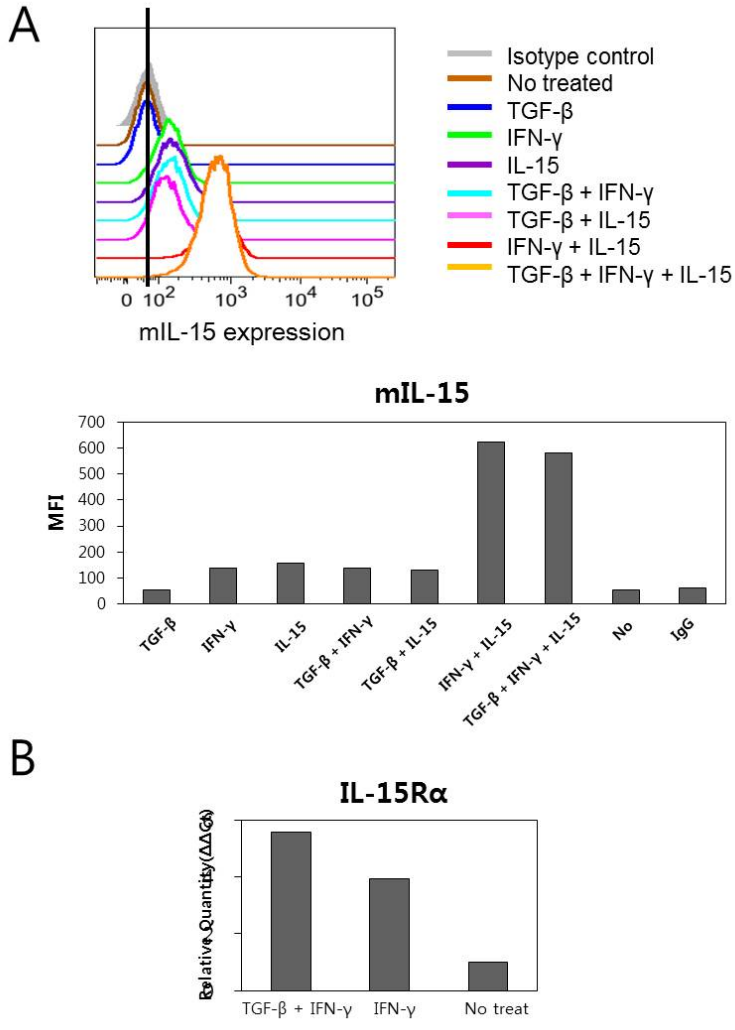


Figure 9. Membrane-bound IL-15 expression on monocytes were elevated by IFN-γ exerting a synergistic effect with IL-15.

(A) Flow cytometric analysis of mIL-15 expression on cytokine-treated monocytes (upper panel). MFI of mIL-15 on cytokine-treated monocytes (lower panel). (B) Relative gene expression of IL-15α (IL-15 receptor α chain) in cytokine-treated monocytes.

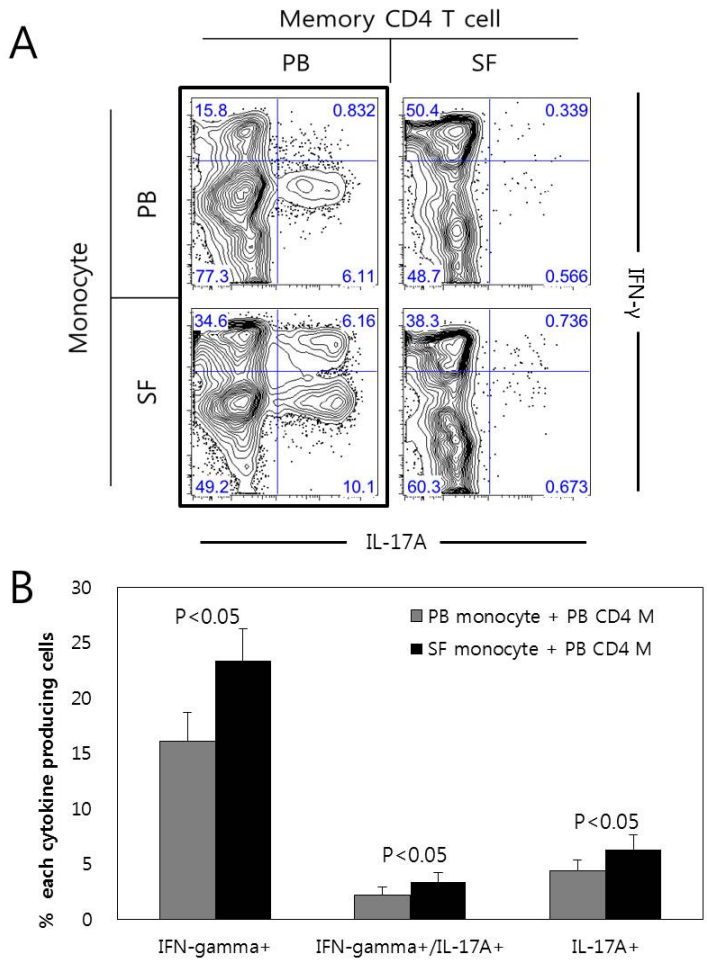


Figure 10. Cytokine production from CD4+ memory T cells after co-culture with monocytes derived from blood or synovial fluid of RA patients.

Isolated monocytes and $CD4^+$ memory T cell from peripheral blood (PB) and synovial fluid (SF) of RA patients were co-cultured with anti-CD3/CD28 and LPS for 7 days as indicated above. On day 7, cells were re-stimulated with PMA and ionomycin in the presence of BFA for 6 hrs and stained with antibodies to IL-17A and IFN-γ after fixation and permeabilization for intracellular cytokine staining (ICS)(n=13).

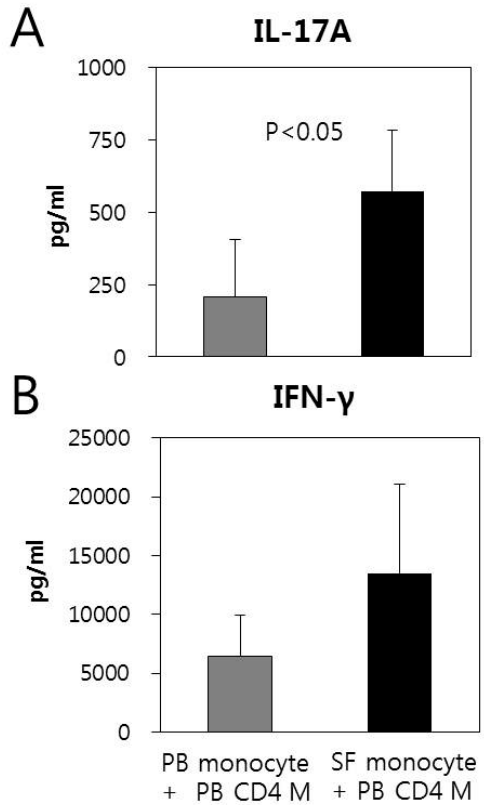


Figure 11. **The synovial monocytes significantly promoted Th17 and Th1 responses *in vitro*, compared with peripheral blood monocytes from RA patients.**

Measurement of IL-17A **(A)** and IFN-γ **(B)** in the culture supernatant were performed by using ELISA (n=6) (NS: not significant)

Discussion

Here I have demonstrated the distinct expression profile of surface molecules on monocytes in RA patients. Compared to healthy controls, PB and SF monocytes from RA patients have increased level of CD16. When co-cultured with PB $CD4^+$ memory T cells from RA patients, significantly enhanced production of IL-17 and IFN-γ were observed in co-culture with SF monocytes but not with PB monocytes in RA patients.

CD16 (FcγRIII) is the type of Fcγ receptor family which is mainly expressed on NK cells and neutrophils. It was well known that cross-linking with IgG antibodies to Fc domain induce various effector functions like phagocytosis, antibody production (ADCC) and production of inflammatory mediators (24, 25). In previous studies, CD16 molecules expressed on $CD14^+$ monocytes in RA patients was suggested to involve in increased responsiveness to immune complex-stimulation (26). The ligation of immune complex to CD16 on monocytes induces TNF-α and IL-1α and these cytokines are involved in the disease process as proved by the clinical efficacy of blocking TNF-α or IL-1 (27, 28). These findings have suggested the possible role of enhanced CD16 level on SF monocytes for inflammatory cytokine milieu of the synovial fluid.

In this study, phenotype analysis with flow cytometry clearly showed that SF monocytes have elevated level of HLA-DR expression and CD80 when compared to PB monocytes, whereas CD86 expressions did not differ between SF and PB

monocyte in RA patients and even in healthy controls. The CD80 and CD86 are known to co-stimulatory molecules which are factors give ‘second signal’ to CD28 or CTLA4 on T cells. CD86 is constitutively expressed on resting monocytes or activated B cells, whereas CD80 is not expressed on resting monocytes (29, 30). In addition, HLA-DR is a MHC class II cell surface molecule that gives ‘first signal’ to TCR(T cell receptor) on CD4 T cells, typically found in antigen presenting cells. Above two signals are necessary to induce immune response of $CD4^+$ T cells, suggesting that the SF monocytes may have distinct phenotype which is suitable for activation of $CD4^+$ T cells.

It is not known yet how and why SF monocytes have higher CD16 expression. Several possible explanations for this phenomenon can be offered; one is that $CD16^+$ monocytes are selectively recruited into inflammatory synovial cavity, other is that circulating “classical” $CD14^+$ monocytes migrate into synovial cavity and change (or differentiate) into “intermediate” or “proinflammatory” $CD14^+CD16^+$ monocytes under certain cytokine milieu of synovial fluid. To test the latter possibility, purified $CD14^+$ monocytes were stimulated with various cytokines and TLR ligands. TGF-β treatment increased the level of CD16 expression on $CD14^+$ monocytes purified from and IFN-γ treatment promotes upregulation of CD80 and mIL-15 levels. However, $CD4^+$ memory T cells of healthy controls co-cultured with artificially modified monocytes expressing higher CD16, CD80, and mIL-15 did not show capability to induce enhanced Th17 and Th1 responses which were markedly observed in the co-culture with PB CD4 T cells and SF monocytes in RA

patients (data not shown). It is suggested that there may be other intrinsic factors of SF monocytes to modulate and shape T cell immune responses.

Collectively, this study demonstrates the phenotypic and functional characteristics of monocytes in SF and PB from patients with RA patients which may be affected by synovial fluid environment having critical roles of pathogenesis of RA. The findings in this study suggest the possible role for cytokine milieu of the SF in giving unique features to synovial monocytes and their important roles in affecting inflammatory T-cell responses in RA.

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국문 초록

단핵구는 감염에 대한 선천성 면역 반응에 관여할 뿐만 아니라, 자가면역질환을 포함하는 만성 염증성 질병의 병인에도 중요한 역할을 한다. 사람 단핵구는 일반적으로 CD14와 CD16의 발현 정도에 따라 세 개의 아형으로 구분한다. 염증성 환경에서 이러한 세 개의 단핵구 아형의 빈도 및 기능변화가 뚜렷한 나타난다는 연구결과들이 증가하고 있음에도 불구하고, 자가면역질환의 병인에서 이들의 역할에 대해서는 알려진 바가 많지 않다. 본 연구에서는 류마티스 관절염 환자에서 얻은 관절활액과 말초혈액 내 단핵구의 표현형과 기능적 차이에 대해 조사하였다. 류마티스 관절염 환자의 활액으로부터 분리한 $CD14^+$ 단핵구에서 CD16 발현이 동일 환자와 건강 대조군의 말초혈액 유래 단핵구에 비해 유의적으로 증가되어있었던 반면에, $CD14^{dim}CD16^+$ 단핵구 비율변화는 활액에서 거의 관찰되지 않았다. 활액 단핵구에서 표면 CD80의 발현이 유의적 증가해 있는데 비하여, 류마티스 관절염 환자 및 정상인의 말초혈액 단핵구에서는 CD80 발현이 관찰 되지 않았다. 또한 막부착형 IL-15의 발현이 류마티스 환자의 단핵구에서 증가해 있었다. 활액 유래 단핵구와 말초혈액 유래 단핵구의 TLR 발현 정도가 상이하였으며, 특히 전반적으로 활액 유래 단핵구의 발현 정도가 더 높게 나타났다. 활액 유래 단핵구가 어떻게 특징적 표현형을 얻게

되는지 알아보기 위하여, 말초혈액 유래 단핵구에 다양한 사이토카인과 TLR ligand를 이용하여 자극하였다. TGF-β가 $CD14^+$ 단핵구로부터 CD16 발현을 유도하는 잠재적 인자였으며, 반면 CD80과 막부착형 IL-15의 발현은 IFN-γ에 의해 유의적으로 증가하였다. 특히 막부착형 IL-15의 경우는 IL-15과 함께 처리하였을 때 상승효과를 확인할 수 있었다. 시험관배양 조건에서, 관절활액 단핵구는 말초혈액 단핵구에 비해 유의적으로 Th17과 Th1의 반응을 증가시켰다. 위 연구들을 통하여 류마티스 관절염 환자의 활액 내 특정 사이토카인 환경이 활액 단핵구의 특징적 표현형을 가지게 하는 데에 역할을 할 수 있음을 확인하였고, 이는 류마티스 관절염 환자의 염증성 T세포 반응에 중요한 영향을 줄 수 있음을 제안하였다.

주요 단어 ; CD16 단핵구, 류마티스 관절염, Th17, TGF-β

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